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Original

Morphological Observation of the Calli derived from Four Coniferous Species *in vitro*

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Abstract——Callus tissues were derived *in vitro* from four coniferous species (*Cryptomeria japonica*, *Pinus thunbergii*, *Ginkgo biloba* and *Sequoia sempervirens*) and the morphological features of the calli were discussed in relation to their growth rates. Xylem and phloem cells differentiated in the calli but these cytodifferentiations were not correlated to growth rate of the calli (summerized in Table 2). On the other hand, the following tissue organizations were correlated to growth rate (summerized in Table 1). High growth rate calli showed a loose intercellular contact and formed no dermal tissue, whereas low growth rate calli formed tight tissues covered with dermal tissues. All calli formed vascular nodules which were categorized into three types, i.e. circular, linear and continuous types. The first one was predominant in high growth rate calli, the frequency of the second one increased as the growth rate became lower, and the third one was formed in the calli which grew extreamly slow. The correlation between tissue organizations and growth rate might be common among species, and the former is controlled by the latter.

Introduction

The cells and tissues differentiated in calli were reported by many investigators. The tracheary elements or wound vessels in angiosperms, often accompanied by reticulate secondary walls, in the *in vitro* systems were reported in coleus[1~3], soybean[4,5], carrot[6,7], etc.. Tracheary elements with bordered pits in gymnosperms were observed in sugi[8], white spruce[9], etc.. Reticulate secondary walls also were observed in tracheary elements of sugi callus[10].

Wetmore and Rier[11] found that some angiosperm-calli formed vascular tissues which consisted of phloem and xylem. Durzan *et al.*[9] observed cambial-like initials which were accompanied by the xylem cells with wall thickenings and bordered pits in *Picea* callus. Aloni[5] reported that low levels of IAA induced sieve elements but no tracheary cells, and that high levels of IAA induced both phloem and xylem in

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calli of *Syringa, Daucus* and *Glicine*. Yamamoto *et al.*[8] observed cylindrical and nodular vascular tissues in the callus derived from the xylem core segments of sugi trunks *in vitro*. These tissues described above are tentatively named as " vascular nodule " in the present paper.

Morphological features of cultured tissues vary among species. Such variations are comparable with each other only when tissues are cultured in the same condition, because the morphological features also vary with culture conditions. However, a certain culture condition for differentiating callus might be suitable only for one species but not for the others. Thus, it is not easy to describe the specific feature of each species in the callus. Moreover, as well as morphological features, growth rates of calli also vary among species in the same culture conditions. Many investigators[3,4,12~18] had paid much attention to the relationship between differentiation of tracheary element and growth rate or cell division. Fukuda *et al.*[12] indicated that cell division is not a prerequisite for tracheary element differentiation. On the other hand, the other morphological features than tracheary element differentiation had scarcely been investigated in relation to cell division or growth rate. If morphological features are compared in relation to growth rates, some relationship between the feature and the growth rate might be revealed. In the present paper, calli of four coniferous species were cultured in the same condition, and their morphological features in relation to the growth rates were compared.

Materials and Methods

Young shoots of sugi (*Cryptomeria japonica*), kuromatsu (*Pinus thunbergii*) and sequoia (*Sequoia sempervirens*), and petioles of ginkgo (*Ginkgo biloba*) were harvested in the summer of 1982 and 1983. These samples were sterilized with 10% NaOCl solution[19]. The sterilized shoot samples and petioles were cut into segments of 5 mm and 10–20 mm long respectively, and were cultured on agar medium (Saito's inorganic medium[20] with amino acids[19], 10 mg/l of indolebutyric acid and 0.02 mg/l of kinetin). Callus tissues, which were formed on the trimmed surfaces of the explants within 1 month-culture, were detouched from the explants and subcultured for another 1 month in the same medium.

The subcultured callus tissues were embedded in paraffin after fixed in FAA solution (Formalin-Acetic acid-Ethyl alcohol), and the serial sections were prepared. These sections were stained with 1% safranin-O and 0.5% fast green-FCF for histological observation. Some sections were stained with the aniline blue according to Currier[21] in order to detect callose. Fine structures were also observed by scanning electron microscopy (HITACHI 450S).

Results and Discussion

1. Morphological Observations of Calli

Morphological observations on sugi, kuromatsu and ginkgo calli are described in the following sections. Sequoia callus will be refered to in the next chapter where calli of the four species are morphologically compared.

1.1 Sugi callus

Sugi callus grew into a mass of about 1 cm in diamter after 2 months-culture. Callus surface was partially covered with newly formed dermal tissue which consisted of phelogen and cork cells with suberized walls (Fig. la, b).

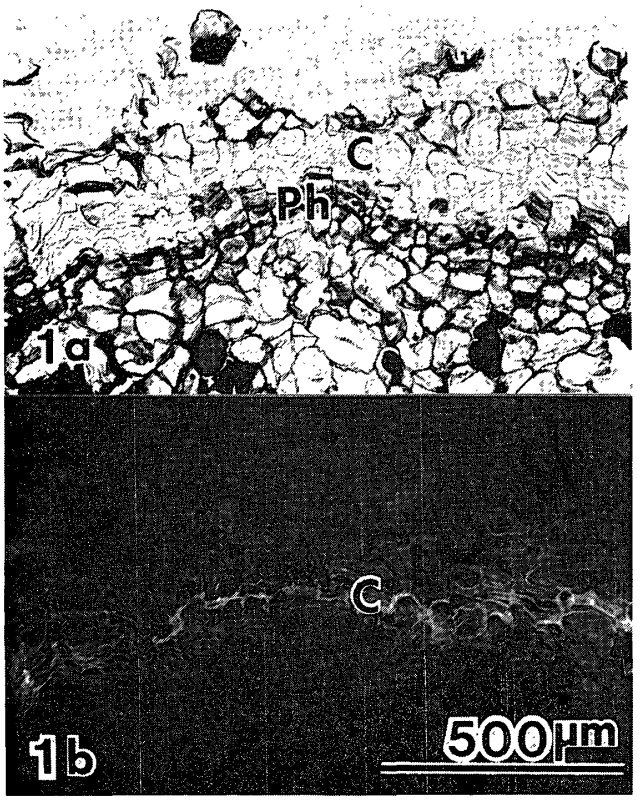


Fig. 1. Light (a) and fluorescence (b) micrographs of dermal tissue of sugi callus. Ph: phelogen, C: cork cells. Suberized walls of cork cells are shown in Fig. 1-b.

Vascular nodules were observed in the callus tissue. They were morphologically varied in a microtomed section, namely linear type (Fig. 2a, b), circular type (Fig. 4a, b) and the intermediate between them (Fig. 3a, b). Each of them was consisted of xylem, mitotic zone and phloem (Fig. 5). The linear type nodule was distributed as a plane along callus surface. The relative location of xylem and phloem observed in these nodules is contrary to the case of normal vascular cambium: xylem outside and phloem inside. In the case of circular nodules, serial sections revealed their glo-

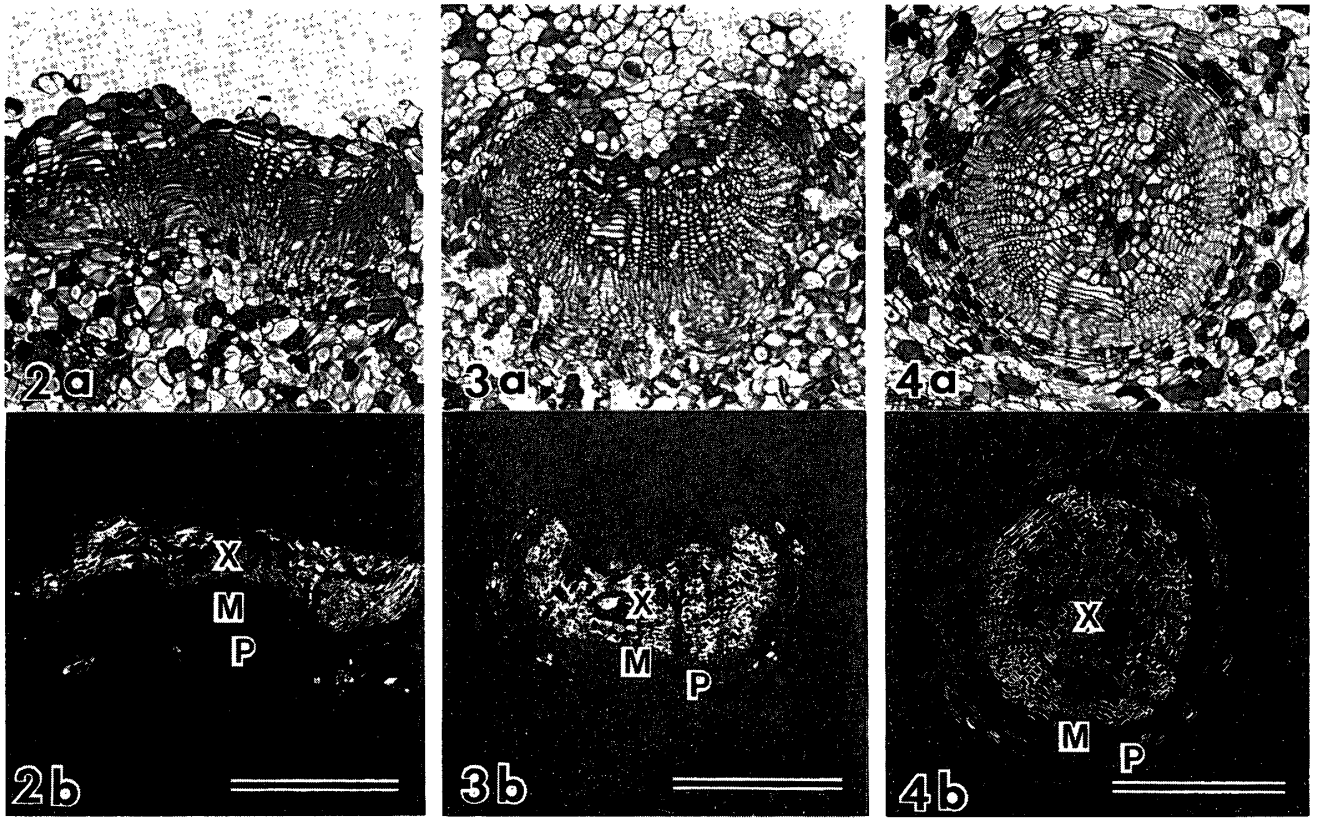


Fig. 2–4. Light (a) and polarization (b) micrographs of vascular nodules of sugi callus. X: xylem, M: mitotic zone, P: phloem (Lines are 500 μm). Fig. 2: linear type, Fig. 3: intermediate type, Fig. 4: circular type.

Fig. 5. A part of sugi vascular nodule which consists of xylem (X), mitotic zone (M) and phloem (P). The mitotic cells show two figures (i.e. slender and rectangular) in the section, because their longitudinal axes made a right angle to each other. In the xylem, two types of tracheary elements, namely slender (SL) adjacent to and spherical (SP) apart from mitotic zone respectively, are shown.

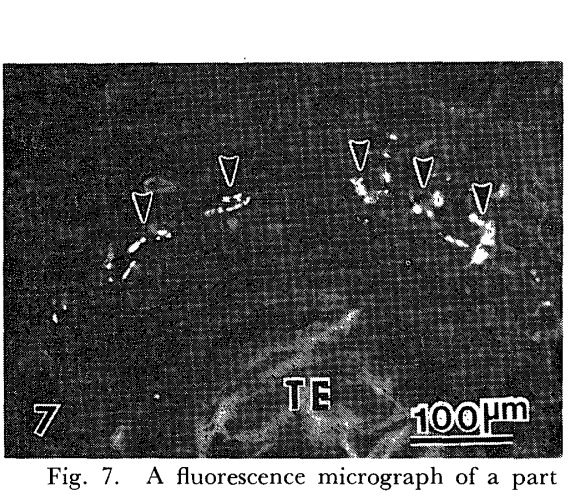
bular shape of about 1 mm in diamter. The xylem was surrounded by the mitotic zone, in the outer region of which phloem cells were distributed. The intermediate nodules between linear- and circular ones apparently look like to be a transitional form between the two types. Generally, if the circular or linear nodule is to be derived from linear or circular one through the intermediate form respectively, these ultimate types should be accompanied by various transitional ones. However, such transitional types were not always observed in the calli of other species than sugi (e.g. only circular ones were formed in ginkgo callus as mentioned later). Therefore, the transition from linear- to circular one, or vice versa, did not seem to occur even in sugi callus. Thus, the intermediate nodule is not a transitional form but considered to be a kind of linear one.

All of the nodules consisted of a few kinds of cells. On the phloem side, two types of cells (i.e. bast fibers and sieve cells) were dispersedly distributed along the mitotic zone. Bast fibers were thick-walled with simple pits (Fig. 6), and sieve cells had sieve areas with callose (Fig. 7, 8).

The mitotic zone consisted of several tiers of thin-walled cells with prominent nuclei. These cells were slender, and their longitudinal axes were oriented in the two different directions group by group, which made a right angle on a tangential plane (Fig. 5).

On the xylem side, there were tracheary elements with secondary walls and

Fig. 6. A SEM micrograph showing a thick walled bast fiber in a vascular nodule of sugi callus.



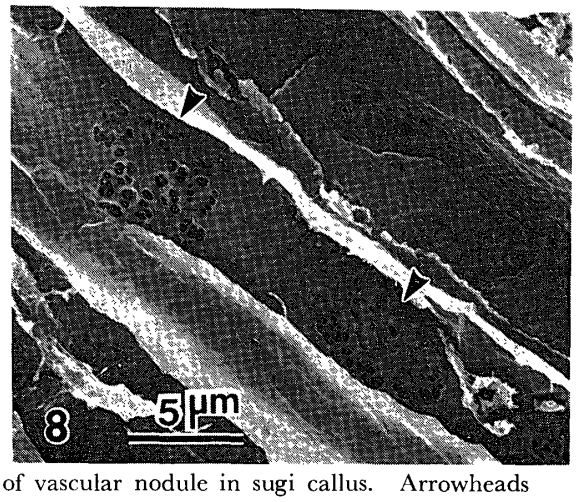


Fig. 7. A fluorescence micrograph of a part of vascular nodule in sugi callus. Arrowheads show callose in sieve cells which stained by aniline blue. TE: tracheary elements with lignified walls.

Fig. 8. A SEM micrograph of a sieve cell with sieve arreas (arrowheads) in a vascular nodule of sugi callus.

bordered-pits (Fig. 9). Some of them were lignified. Neither typical torus nor margo was found in their pits, but residues of middle lamella were frequently observed (Fig. 10). Two types of tracheary elements were formed in the xylem. One is distributed on the xylem side adjacent to the mitotic zone. The tracheary elements in this region were regularly arranged in radial rows and showed slender shapes similarly to mitotic cells (Fig. 5 SL). Their walls were gradually thickened and lignified with the distance from mitotic zone. These observations suggested that they were derived

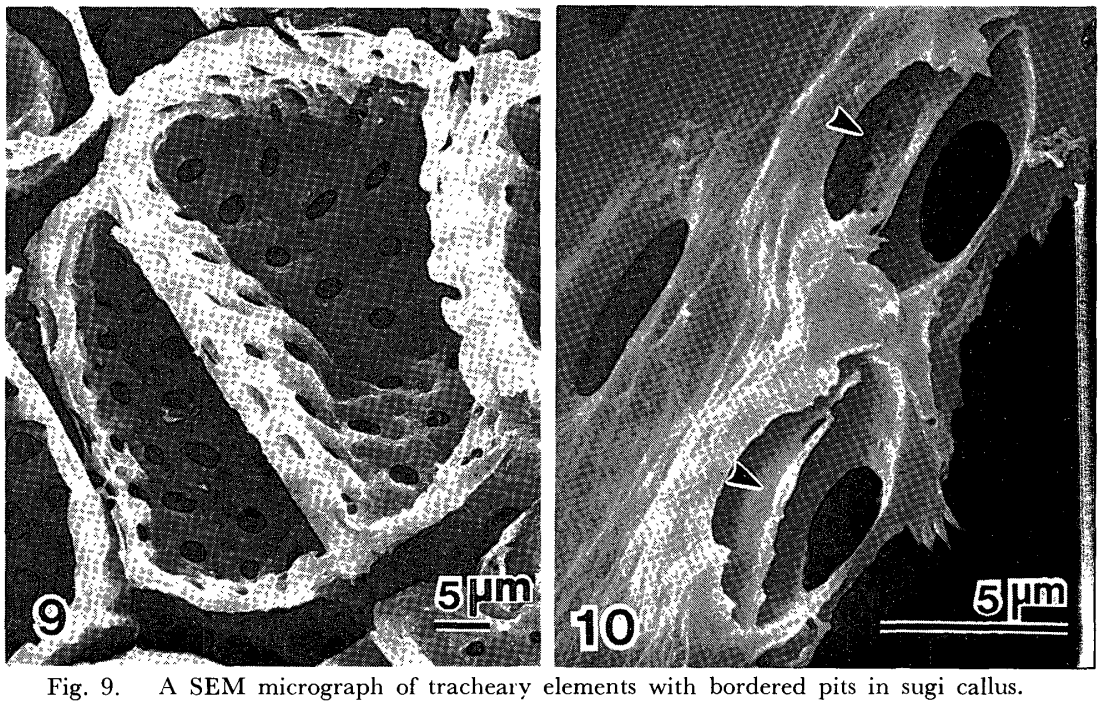


Fig. 9. A SEM micrograph of tracheary elements with bordered pits in sugi callus.
Fig. 10. A SEM micrograph showing bordered pits of tracheary elements in sugi callus. Arrowheads show residues of middle lamella lacking typical torus and margo.

from mitotic zone. The other tracheary elements were located in the xylem apart from mitotic zone. They were irregularly arranged and their shapes were spherical (Fig. 5 SP). They were not always lignified and frequently formed reticulate walls. Tracheary elements of this type were also distributed in the areas where vascular nodules were not fully developed nor formed. These observations suggest the order of the development of vascular nodules, i.e. the spherical tracheary elements might be differentiated in a callus tissue at first and then the cells might be surrounded by a mitotic zone which produces slender ones.

1.2 Kuromatsu callus

Kuromatsu callus showed a highly tight intercellular contact and formed more rigid tissues comparing with the case of sugi callus. The callus was perfectly covered with dermal tissue, in which the cells with suberized walls were observed, although phelogen was not formed.

The vascular nodule extended along the callus surface and surrounded parenchymatous callus core (Fig. 11, 12). It is apparently similar to the linear type as described in sugi callus, except the relative location of xylem and phloem, i.e. xylem inside and phloem outside. Based on the observation of the nodule-shape by serial sections, the nodule was traced to have been connected with cambium of the mother explant before the callus tissue was detouched. Such a type of nodule was categorized as a continuous nodule. This nodule was similar to a regenerating cambium in

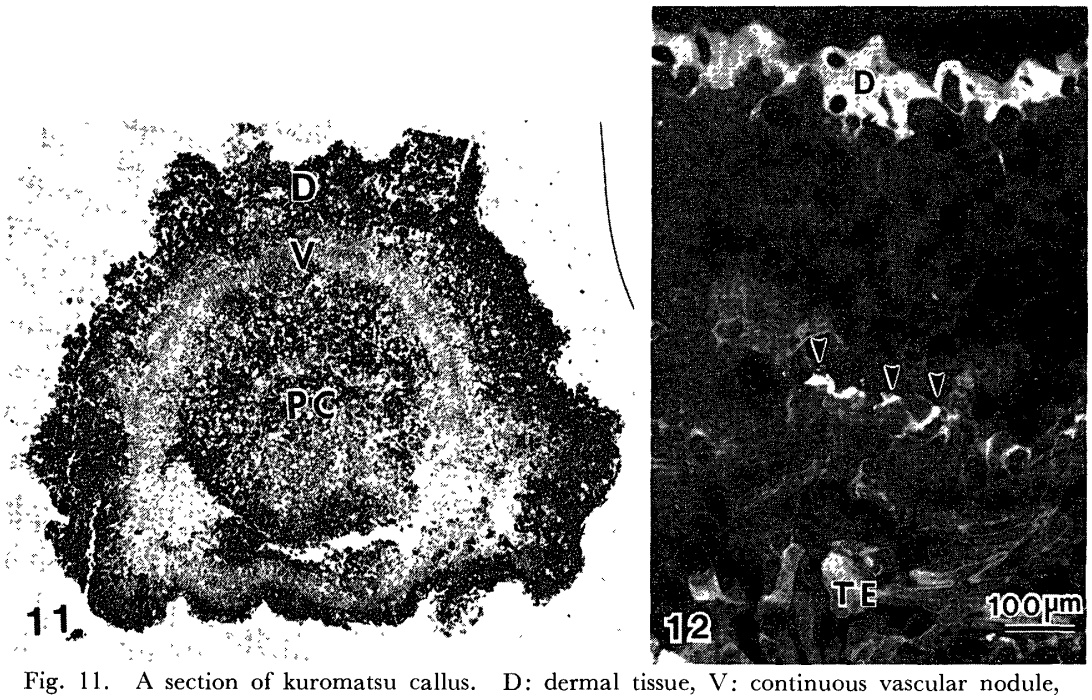


Fig. 11. A section of kuromatsu callus. D: dermal tissue, V: continuous vascular nodule, PC: parenchymatous callus core. The dermal tissue perfectly covered callus tissue and continuous nodule surrounded parenchymatous core.

Fig. 12. Fluorescence micrograph of a part of kuromatsu callus. Arrowheads show callose stained with aniline blue in phloem. D: suberized dermal cells which covered callus surface. TE: tracheary elements with lignified walls in xylem. The xylem and pholem were respectively located in the inner and the outer side of callus.

the wound stem.[22] " A cylindrical vascular tissue ", which was observed by Yamamoto *et. al.*, would be also categorized into this nodule.

1.3 Ginkgo callus

Ginkgo callus formed the most loose intercellular contact among the species examined. The callus was easily disassembled into single cells or small cell clumps.

A small nodule was often observed in a small mass of callus (Fig. 13). They carried several tracheary elements in the center, some of which were stained weakly with safranin. No regularly oriented mitotic zone was found, but parenchymatous cells surrounded the tracheary elements in the nodules. Neither sieve cells nor bast fibers were observed.

Two types of cells were observed specifically in ginkgo callus. One is the cell which contained a lot of starch grains (Fig. 14). Starch grains were observed also in the calli of other species examined, but they were not abundant comparing with those of ginkgo callus. The other is the cell which contained a crystal reminiscent of the idioblast in the ginkgo stem (Fig. 15). Tulecke[23] also observed such crystal inclusions in ginkgo tissue cultures.

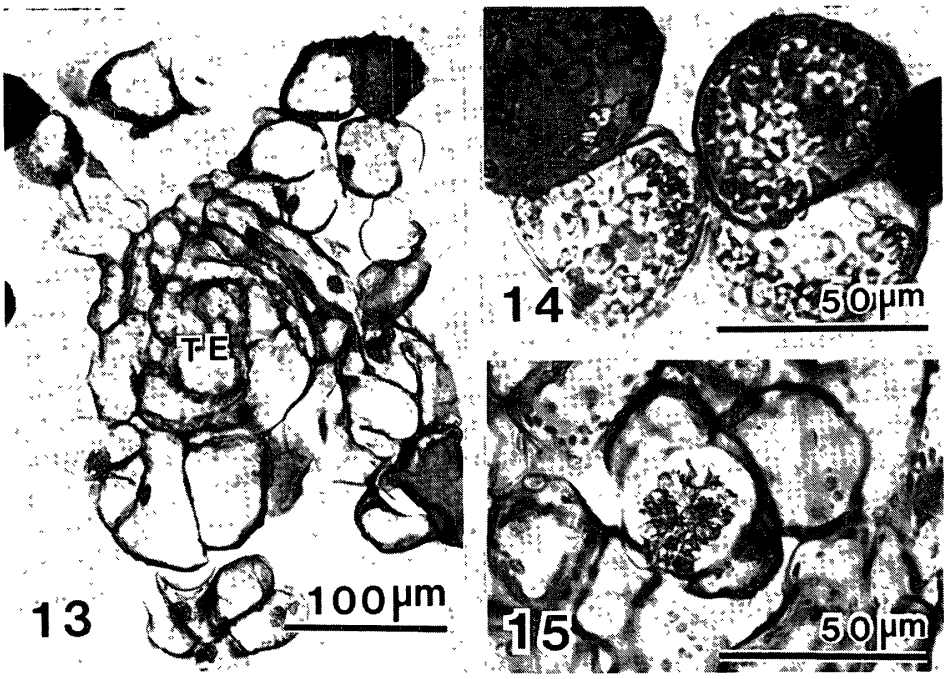


Fig. 13. Vascular nodule in ginkgo callus. Tracheary elements (TE) are surrounded by parenchymatous cells (details see in the text).
Fig. 14. Cells containing a lot of starch grains in ginkgo callus.
Fig. 15. A cell containing a crystal in ginkgo callus.

2. Morphological Comparison of Calli formed in the Four Species

The calli derived from the four species showed various growth rates and morphological features, although they were cultured in the same condition. The morphological features prominent enough to discuss were tissue organization and cytodifferentiation. They will be summarized in this chapter in relation to the growth rate.

2.1 Tissue organization in calli

Tissue organization in calli was correlated to the growth rate as shown in Table 1. Four species in the table are arranged in the order of growth rates, i.e. ginkgo, sequoia, sugi and kuromatsu calli, successively.

Table 1. Growth rate and tissue organization in four coniferous calli

		Ginkgo	Sequoia	Sugi	Kuromatsu
	Growth rate	****	***	**	*
Tissue organization	Tightness	+	++	+++	+++
	Dermal tissue	−	−	+	++
	Type of nodule	CI	CI, (L)	CI, L	CO
	Regularity of cell arrangement in nodule	+	++	+++	+++
	Development of nodule	+	++	+++	+++

*: degree of growth rate, +: degree of tissue differentiation, −: none, CI: circular nodule, L: linear nodule, CO: continuous nodule (details see in the text).

Callus formed tight tissues when growth rate was low, while a high growth rate callus showed a loose intercellular contact. The growth rate might control tightness of the callus tissues. Dermal tissue was not recognized in the high growth rate callus. Accordingly, sugi callus partially formed dermal tissue, and kuromatsu callus, which showed the lowest growth rate, was parfectly covered with dermal tissue. Therefore, dermal tissue formation seemed to be controlled by the growth rate. The growth rate was also correlated with the three types of nodule formation, i.e. circular-, linear- and continuous nodules (intermediate nodule was categorized into linear type). The circular nodules were predominant in the high growth rate callus, whereas the frequency of linear nodules increased as the growth rate became lower, and the continuous nodule was formed in the callus which grew at extreamly low rate. Cells were regularly arranged in nodules when the growth rate was low. Nodules were highly developed in the low growth rate.

The tissue organization was correlated to growth rate of calli as shown in Table 1. If the growth rate of callus is controlled by culture conditions, the tissue organization might be varied even within a species according to the tendency shown in Table 1. Actually, when sugi callus was cultured in the medium with additional kinetin (0.2–5 mg/l), it grew more slowly, and formed a more tight tissue which was perfectly surrounded by a dermal tissue. It even formed continuous nodule as kuromatsu callus did. Therefore, the potentialities for differentiating any of these tissue organizations are common among species, but they are controlled by the growth rate specific to each species. Some investigators[24~26] proposed an idea that morphogenesis might be controlled by a microenvironment orderly distributed in a plant tissue. If such an idea is true, our results would be interpreted as follows: Distribution of the microenvironment would be distributed by expansion of a callus tissue. Thus, the callus tissues were less organized when the callus grew fast and were extremely expanded. On the other hand, distribution of microenvironment would be hardly disturbed in the low growth rate callus, and the tissues were highly organized.

2.2 Cytodifferentiation in calli

Table 2 shows morphological features concerning the cytodifferentiation in the calli of the four species. Tracheary elements differentiated in all of the four species, but the extent of their differentiation varied conspicuously without any correlation to growth rate, as shown in Table 2. The sugi cell walls were highly thickened and lignified. In ginkgo, tracheary elements were poorly stained with safranin and formed thinner secondary walls than that in sugi. In sequoia and kuromatsu, tracheary elements were not stained with safranin, but a slight fluorescence caused by lignin was observed under a fluorescence microscope. Callose was observed only on the phloem side of the nodule in sugi and kuromatsu callus, while it was also distributed in the mitotic

Table 2. Growth rate and cytodifferentiation in four coniferous species

		Ginkgo	Sequoia	Sugi	Kuromatsu
	Growth rate	****	***	**	*
Cytodifferentiation	Tracheary element differentiation	+	+	+	+
	wall thickness	+	+	++	+
	lignification	++	+	+++	+
	Sieve cell	−	+	+	+
	Bast fiber	−	−	+	−

*: degree of growth rate, +: presence or degree of development, −: none (details see in the text).

zone and xylem in sequoia. Callose was hardly observed in ginkgo. Bast fibers were observed only in sugi callus.

Fukuda and Komamine[27] proposed that " the process of cytodifferentiation is independent of the progression of the cell cycle", from the result of the mesophyll single cell culture of *Zinnia elegans*. Our result that the cytodifferentiation was not correlated to callus growth rate or cell division as shown in Table 2 is consistent with their proposal.

The calli derived from the four species showed various morphological features, (Table 1, 2) even though they were cultured in the same medium. One of the reasons is naturally ascribed to the genetically controled specific character of each callus. The second reasson is that their respective features might be affected by physiological conditions exuded from their respective explants, because the calli had attached to the explants for the first one month. The third reason is due to the difference in the expansion of callus tissue at each growth rate, which might disturb ordered distribution of morphogenetical microenvironment.

We compared these morphological features in relation to growth rate and found that tissue organization was correlated to growth rate but cytodifferentiation was not. The growth rate and cytodifferentiation would be affected by culture conditions, as well as genetically specific characters of each species, while the growth rate, in turn, seemed to control the tissue organization.

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